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Genetic Characterization of the Vaccinia Virus DNA Polymerase: Identification of Point Mutations Conferring Altered Drug Sensitivities and Reduced Fidelity

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We determined that 85 μM aphidicolin was sufficient to block macroscopic plaque formation by vaccinia virus and to cause a 10^4-fold reduction in viral yield from a wild-type infection. A chemically mutagenized viral stock was passaged sequentially in the presence of drug, and plaque-purified viral stocks resistant to aphidicolin were isolated and characterized. By use of a marker rescue protocol, the lesion in each mutant was found to map within the same 500-bp fragment within the DNA polymerase gene. All of the mutants were found to contain a single nucleotide change in the same codon. In nine of these mutants, the alanine residue at position 498 was changed to a threonine, whereas a 10th mutant sustained a valine substitution at this position. Congenic viral strains which carried the Aph^r lesion in an unmutagenized wild-type background were isolated. The Thr and Val mutations were found to confer equivalent levels of drug resistance. In the presence of drug, viral yields were 25% of control levels, and the levels of viral DNA synthesized were 30 to 50% of those seen in control infections. The two mutations also conferred an equivalent hypersensitivity to the cytosine analog 1-β-D-arabinofuranosylcytosine (araC); strains carrying the Thr mutation were moderately hypersensitive to the pyrophosphate analog phosphonoacetic acid and the adenosine analog araA, whereas the Val mutation conferred acute hypersensitivity to these inhibitors. The Val mutation also conferred a mutator phenotype, leading to a 20- to 40-fold increase in the frequency of spontaneous mutations within the viral stock.

Vaccinia virus displays an unusual degree of physical and genetic autonomy from host cell functions. Indeed, the virus is presumed to encode all of the proteins required for replication and transcription of the 192-kb DNA genome. The genetic complexity and cytoplasmic localization of the virus make it a superb model system for detailed studies of nucleic acid metabolism. Pursuant to our efforts to study the replication machinery encoded by vaccinia virus, we have undertaken the isolation and characterization of viral mutants with conditionally lethal and drug-resistant lesions in the DNA polymerase.

The 1,006-amino-acid DNA polymerase contains polymerase and proofreading exonuclease activities and retains the conserved sequences shared among numerous viral (phi-29, T4, adenovirus, vaccinia virus, herpes simplex viruses types 1 and 2 [HSV-1 and -2], Epstein-Barr virus, and cytomegalovirus) and eucaryotic (human α and yeast pol I) polymerases (3, 4, 12, 24, 27, 43, 44). These homologies are thought to mark functional domains involved in template or substrate binding and/or catalytic activity. To date, two temperature-sensitive (codons 392 and 611), one phosphonoacetic acid-resistant (PAA^r), and one aphidicolin-resistant (Aph^r) lesion have been localized to specific codons of the viral polymerase (10, 12, 38, 39). The repeated recovery of the same PAA^r lesion (codon 372, G → D) in several independent selections suggests that this lesion may identify the site of the enzyme's interaction with PAA, an inhibitor of pyrophosphate exchange. Aphidicolin, a fungal tetracyclic diterpenoid, is a specific inhibitor of members of the DNA polymerase α family. Its inhibitory action stems from its presumed interaction with nucleotide-binding domains; biochemical studies have indicated that aphidicolin acts as a competitive inhibitor with respect to dCTP and a noncompetitive inhibitor with respect to dATP, dGTP, and TTP in its interactions with the viral enzyme (34). The one known Aph^r lesion identified within a mutated vaccinia virus DNA polymerase (codon 670) (10) maps within conserved domain III shared by various replicative polymerases (43, 44). In light of the close association of aphidicolin with the nucleotide-binding domain of several polymerases, we have pursued the isolation of additional Aph^r mutants in an effort to probe the interaction of the viral polymerase with its substrates.

MATERIALS AND METHODS

Cells and virus. Mouse L cells, African green monkey BSC40 cells, and wild-type (wt) vaccinia virus (WR strain) were propagated as previously described (13, 39). Aphidicolin-resistant virus was selected from a nitrosoguanidine-mutagenized stock of the wt virus, as described below. Propagation of these mutants and all virological assays were carried out on monolayers of BSC40 cells. All virus stocks were stored at −80°C.

Materials. Restriction endonucleases, calf intestinal phosphatase, *Escherichia coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, polynucleotide kinase, and pancreatic RNase were obtained from New England BioLabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the instructions of the manufacturer. ^{32}P-labeled nucleotide triphosphates were obtained from New England Nuclear Corp. (Boston, Mass.). Aphidicolin, PAA, rifampin, cytosine β-D-arabinofuranoside (araC), adenosine β-D-arabinofuranoside (araA), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were purchased from

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Sigma Chemical Co. (St. Louis, Mo.). Isatin β-thiosemicarbazone (IBT) was obtained from Pfaltz & Bauer, Inc. (Waterbury, Conn.). Aphidicolin and rifampin were prepared as 10 mM and 25-mg/ml stocks, respectively, in dimethyl sulfoxide and stored at −20°C. PAA was prepared as a 10-mg/ml stock in distilled water, adjusted to pH 7.0, and stored at 4°C. AraC and araA were prepared as 1 mM stocks in phosphate-buffered saline (PBS), pH 7.4, and stored at −80°C. MNNG was prepared as a 1-mg/ml stock in 10 mM Tris buffer, pH 7.3, and stored at −20°C. IBT was prepared fresh just before use by first dissolving it in acetone to 5 mg/ml and then diluting it to 1 mg/ml with 4 volumes of 0.25 M NaOH. Each drug was diluted to its final concentration in Dulbecco modified Eagle medium (DMEM) with 5% fetal calf serum (FCS) just before use.

Determination of viral yields. Viral titers were determined by plaque assay as follows. Following at least two cycles of freeze-thaw to release virus from infected cells and two 15-s bursts of sonication, virus preparations were serially diluted in DMEM and plated on monolayers of BSC40 cells. Following a 1-h adsorption period, the inoculum was removed and the cells were fed with DMEM–5% FCS with or without any drugs. After 48 h of incubation at 37°C, the plates were fixed and stained with 0.1% crystal violet, and plaques were counted manually.

Mutagenesis. Confluent monolayers of BSC40 cells (150-cm^2 flasks) were infected with wt virus at a multiplicity of infection (MOI) of 10 PFU per cell. After 30 min of adsorption at 32°C, the inoculum was removed and the monolayers were rinsed once with DMEM and fed with DMEM–5% FCS containing MNNG at 5 μg/ml. After 10 h of incubation at 32°C, the medium was removed. The cells were then rinsed twice with DMEM, fed with MNNG-free medium, and returned to 32°C (8). At 48 h postinfection (p.i.), cells were scraped from the flask, collected by centrifugation, and resuspended in 2 ml of PBS. Virus was released from cells by two cycles of freeze-thaw and stored at −80°C. For comparative purposes, a nonmutagenized control stock was similarly prepared.

Isolation of aphidicolin-resistant mutants. Mutants were selected by serial passage of mutagenized virus through BSC40 cells in the presence of 85 μM aphidicolin at 32°C. The initial passage was performed by infecting a BSC40 monolayer with mutagenized vaccinia virus at an MOI of 0.5; following a 60-min adsorption period, the inoculum was removed and the monolayer was fed with DMEM–5% FCS containing 85 μM aphidicolin. After 3 days of incubation, cells were scraped from the plates, collected by centrifugation, and resuspended in 1 ml of PBS. Virus was released from cells by two cycles of freeze-thaw. Three subsequent passages were performed in this manner, except that (i) the inoculum for passages 2 and 3 consisted of one-fifth of the harvest of the previous passage, which in retrospect corresponded to MOIs of 0.1 and 0.3, respectively; (ii) passage 4 was performed at an MOI of 0.15; and (iii) incubation of passages 2, 3, and 4 was for 5 days. Yields of total and aphidicolin-resistant virus from each passage were determined by plaque assay at 37°C in the absence or presence of aphidicolin, respectively.

The yield from passage 4 was used as the inoculum for plaque purification of several drug-resistant mutants. BSC40 monolayers were infected with approximately 10 PFU from passage 4 and overlaid with DMEM–5% FCS containing 0.8% agarose and 85 μM aphidicolin. After 6 days of incubation at 32°C, monolayers were stained with 0.01% neutral red in PBS for several hours. Following removal of the neutral red solution, well-isolated plaques were picked with sterile pasteur pipettes and suspended in 100 μl of PBS. Following two cycles of freeze-thaw and two 15-s bursts of sonication, plaque suspensions were used to infect fresh monolayers for a second plaque purification under agarose. Well-isolated plaques were picked and stored as described above. Ten such isolates (AR *n*) were amplified on BSC40 cells in the presence of aphidicolin. Cells were scraped from 15-cm dishes, collected by centrifugation, and resuspended in 1 ml of PBS. The titer of each stock was determined by plaque assay in the presence or absence of aphidicolin.

DNA preparation and molecular cloning. Genomic DNA from each Aph^r isolate was obtained by harvesting monolayers of infected cells at the point of maximal cytopathic effect. After lysis of the cells by hypotonic swelling, the nuclei were removed by low-speed centrifugation and the viral cores were then collected by sedimentation. Viral DNA was then purified as described elsewhere (39) and digested with restriction endonucleases according to standard procedures. DNA fragments fractionated on agarose gels cast and run in TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA) were isolated by glass powder purification (42) and cloned into pUC8 or pUC19 by standard procedures. The wt *Hin*dIII-E clone was kindly provided by B. Moss. Plasmids were grown in *E. coli* HB101 cells under ampicillin selection and were purified by the alkaline lysis procedure (22).

Marker rescue. Confluent monolayers of BSC40 cells (35-mm tissue culture dishes) were infected with wt virus at an MOI of 0.03 at 37°C. At 4 h p.i., linearized plasmid DNA (~3.5 μg) was added in the form of a calcium phosphate precipitate (13). At 15 h p.i., aphidicolin at 85 μM was added to the dishes. (For rescues in which 500-bp DNA fragments were used, the precipitate-containing medium was removed and monolayers were rinsed once with fresh DMEM–5% FCS prior to being refed with DMEM–5% FCS containing 85 μM aphidicolin.) Plates were incubated an additional 2 or 3 days at 37°C and then harvested and titrated as described above.

Isolation of viral isolates carrying the individual aphidicolin-resistant mutations in a wt genomic background. Plaque-purified Aph^r isolates were obtained from wt-infected monolayers individually transfected with the minimal 500-bp fragment cloned from the genomes of five Aph^r mutants and shown to carry the Aph^r lesion (see above and Table 1). Serial dilutions of these crude marker rescue stocks were applied to monolayers, and Aph^r isolates were obtained by plaque purification under agarose at 37°C in the presence of aphidicolin. From each of the five infection-transfections, two well-isolated plaques were picked, subjected to a second round of plaque purification, and amplified through BSC40 cells to generate crude viral stocks (extracts from infected monolayers, 15-cm-diameter dishes). The second plaque purification and subsequent amplification of the virus was done in the presence of aphidicolin for one isolate (ARP-*n*+) from a marker rescue and in the absence of aphidicolin for the other isolate (ARP-*n*) from that rescue.

DNA sequencing. Plasmids were digested with the appropriate restriction enzyme and then either end labeled at the 3′ terminus with the Klenow fragment of DNA polymerase I and the appropriate α-^{32}P-deoxynucleoside triphosphate or at the 5′ terminus with polynucleotide kinase and [γ-^{32}P]ATP after dephosphorylation with calf intestine alkaline phosphatase. Radiolabeled DNA was then subdigested to yield a DNA fragment labeled at a single terminus; fragments were purified by glass powder purification after agarose gel electrophoresis. Labeled fragments were sequenced by the

Maxam-Gilbert chemical degradation procedure (30). Except where indicated, sequences were obtained for both strands.

Determination of viral yield in single-step growth experiments. Viral yields from a single round of infection were determined as follows. Confluent dishes of BSC40 cells were infected with wt or mutant virus at an MOI of 0.3 or 1.0, as indicated in the figure legends. Following a 1-h adsorption period in DMEM at 37°C, the inoculum was removed and monolayers were fed with DMEM–5% FCS containing various concentrations of aphidicolin, PAA, araC, or araA. At 24 h p.i., cells were harvested by scraping plates with a rubber policeman, collected by low-speed centrifugation, and resuspended in 10 mM Tris buffer (pH 9.0). Following two cycles of freeze-thaw to release virus from the cells, viral yields were determined by plaque assay in the absence of drugs.

Quantitation of viral DNA replication by dot blot hybridization. Confluent 35-mm dishes of BSC40 cells were infected with wt or mutant virus at an MOI of 5 PFU-cell. After a 30-min adsorption period at 37°C, the inoculum was removed and cultures were rinsed and fed with DMEM–5% FCS with or without aphidicolin at 85 μM and then incubated for various times at 37°C. Cell lysates were then prepared, blotted to Zeta-probe membranes (Bio-Rad Laboratories, Richmond, Calif.), and hybridized with viral probes prepared by nick translation (36) as previously described (35).

RESULTS

Dose-dependent inhibition of vaccinia virus replication by aphidicolin. In order to optimize conditions for the isolation of resistant viral isolates, the dose dependency of the inhibitory effect of aphidicolin was monitored. Plaque assays were performed in the presence of a range of concentrations (5 to 135 μM) of aphidicolin, and plaque sizes were compared with those in control assays; a total absence of macroscopic plaque formation was evident at 65 μM aphidicolin. A more quantitative assessment of the impact of aphidicolin on viral replication was obtained by titrating the virus harvested 24 h after infection of confluent monolayers at an MOI of 0.3. As shown in Fig. 1A, aphidicolin exerted a dramatic and dose-dependent inhibition of viral replication; in the presence of 75 or 90 μM drug, 0.005 or 0.0005% of control levels of virus was produced, respectively. Based on these two assays of drug efficacy, a dosage of 85 μM aphidicolin was chosen for further studies.

Isolation of Aph^r virus from a mutagenized viral stock. Wt vaccinia virus was mutagenized in vivo with nitrosoguanidine as previously described (8); briefly, confluent monolayers containing 4×10^7 cells were infected at an MOI of 10 and exposed to the mutagen during part of the replicative cycle. Although this level of mutagenesis had only a minimal effect on virus viability as assayed by yield, the success of the mutagenesis procedure was evidenced by the 110-fold increase observed in the frequency of PAA^r mutants following mutagenesis (data not shown). Within the mutagenized stock, the frequency of viruses with resistance to the chosen dose of 85 μM aphidicolin was 5×10^4 PFU/1.4×10^9 total PFU, or $3.6/10^5$ PFU. By comparing these titrations with those of unmutagenized stocks, we can estimate that a minimum of 98.7% of these resistant viruses were generated during the mutagenesis procedure. If one makes the oversimplifying assumptions that each infected cell produced an equivalent yield of virus following mutagenesis and that each individual cell's yield was homogeneous, then these calculations imply that Aph^r isolates were generated in approximately 1,440 independent events, each of which yielded 70 sibling viruses.

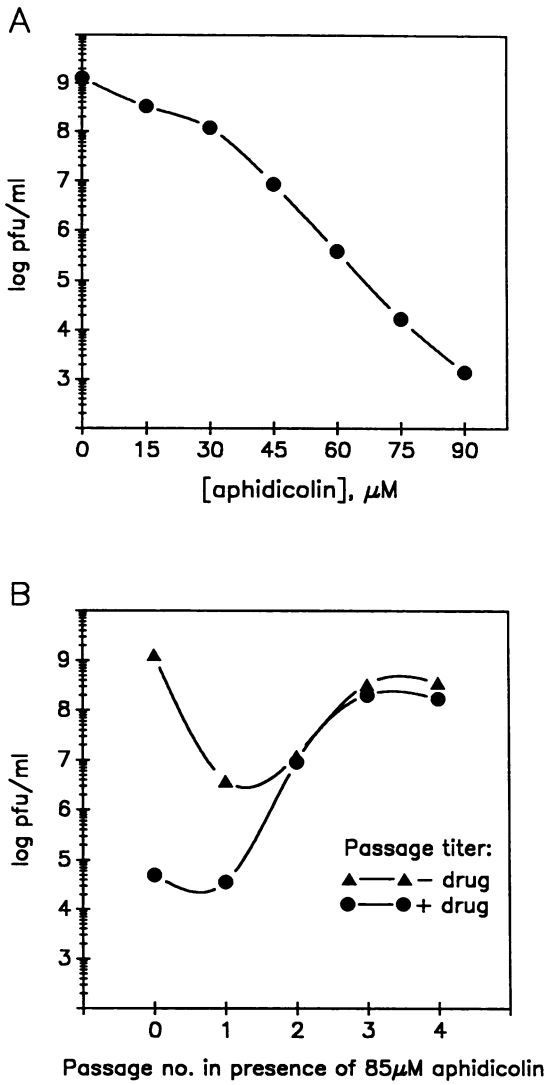

FIG. 1. (A) Dose-dependent inhibition of vaccinia virus replication by aphidicolin. BSC40 cells were infected with wt virus at an MOI of 0.3 and fed with media containing various concentrations of aphidicolin. Virus was harvested at 24 h p.i. and titrated in the absence of drug as described in Materials and Methods. (B) Isolation of Aph^r virus by repeated passaging in the presence of drug. BSC40 cells were infected with MNNG-mutagenized virus (passage 0) at an MOI of 0.5 and fed with medium containing 85 μM aphidicolin. At 3 days p.i., infected cells were harvested and designated passage 1 cells. Three further passages were performed as described in Materials and Methods. Yields of total (▲) and aphidicolin-resistant (●) virus from each passage were determined by plaque assay in the absence and presence of aphidicolin, respectively.

The strategy we employed to isolate several Aph^r isolates was repeated passaging in the presence of drug. The mutagenized stock was used to infect a confluent monolayer of BSC40 cells at an MOI of 0.5; this initial inoculum contained approximately 180 Aph^r viruses. Harvesting of virus from this infection and subsequent passaging were performed as

described in Materials and Methods. The total and Aph^r viral yields from four sequential passages in the presence of drug are shown in Fig. 1B. By the second passage, significant levels of resistant virus were apparent; subsequent passages yielded a stock of high titer which was equivalent in the absence or presence of drug. Ten doubly plaque purified isolates were obtained from the fourth passage; five of these isolates were chosen for extensive study. Stocks prepared from the AR 5, 8, 9, 11, and 12 isolates displayed equivalent titers in the absence or presence of 85 μM aphidicolin (data not shown).

Mapping of Aph^r lesions within the DNA polymerase gene. Genomic DNA was prepared from each of the drug-resistant mutants, and production of Aph^r progeny upon infection with wt virus and transfection with these DNA preparations was confirmed. During these preliminary studies, we determined that inclusion of aphidicolin at the time of transfection (4 h p.i.) was extremely inhibitory to either uptake or expression of the transfected DNA. Addition of the drug at 15 h p.i. alleviated this inhibition while exerting sufficient selective pressure to support the preferential spread of drug-resistant virus during the remainder of the incubation period.

The ability of transfected, subgenomic DNA fragments to undergo homologous recombination with the vaccinia virus genome during infection was then exploited to map the mutations responsible for the Aph^r phenotype. Because the DNA polymerase gene was the most likely locus for aphidicolin resistance, plasmids containing the *Hin*dIII E fragment (15 kb) or its right half (*Bam*HI-*Hin*dIII, 7.5 kb) were cloned directly (40). Both of these fragments include the entire polymerase gene, and as shown in Table 1, the *Bam*HI-*Hin*dIII fragments from each of the five mutants conferred aphidicolin resistance on a significant proportion of the viral progeny during a marker rescue experiment. A series of overlapping or contiguous clones derived from the polymerase gene were then prepared as shown in Fig. 2. Since the overlapping M2700 and R2900 fragments each conferred aphidicolin resistance (Table 1), we concluded that in all cases the lesions mapped within the common 1,400 nucleotides delimited by the *Eco*RI and *Mlu*I sites. For each mutant, this map assignment was refined by testing the ability of the contiguous *Eco*RI-*Hae*III, *Hae*III-*Hin*cII, and *Hin*cII-*Hae*III fragments to confer drug resistance (Fig. 2). In each case, only the central *Hae*III-*Hin*cII fragment was successful in this assay (Table 1).

The complete DNA sequences of the 500-bp *Hae*III-*Hin*cII fragments derived from the five isolates were determined. The results are shown in Fig. 3. In each case, the sole divergence from the wt DNA polymerase sequence was a single nucleotide change within the codon responsible for amino acid 498 of the vaccinia virus DNA polymerase (12, 38, 39). In AR 5, 8, 11, and 12, identical G-A transitions in the first position (nucleotide 1492) were seen. These transitions were predicted to direct the substitution of a threonine for the wt alanine. In AR 9, a C-T transition observed in the second position (nucleotide 1493) resulted in the substitution of a valine for the wt alanine. Because of the surprising redundancy of the mutations, partial sequence data were obtained for the other five aphidicolin-resistant isolates (AR 3, 6, 7, 10, and 14). These five isolates all contained the G-A transition at nucleotide 1492 seen in AR 5, 8, 11, and 12.

Preliminary phenotypic analyses of Aph^r mutants: determination of mutation rate. It has been observed with other systems that genetic resistance to polymerase inhibitors presumed to interact with the nucleotide-binding domain is sometimes associated with altered polymerase fidelity (17, 18, 20, 28, 32). In this context, the Aph^r isolates were investigated for alterations in mutation rate by assaying the spontaneous frequency of viral mutants resistant to PAA, IBT, and rifampin. IBT causes a degradation of mRNA and rRNA during the late stages of poxvirus infection (33), whereas rifampin inhibits polypeptide-processing events associated with virion morphogenesis (16, 23). Resistance to each of these three drugs has been associated with nucleotide changes in individual genes (1, 12a, 37). In duplicate experiments, wt and Aph^r stocks were titrated in the pres-

TABLE 1. Mapping of lesions conferring aphidicolin resistance by marker rescue with cloned subfragments of five mutant DNA polymerase alleles

DNA fragment[a]	Titer of yield (PFU/ml)[b] with:		Rescue
	No drug	Aphidicolin (85 μM)	
No DNA	1.8×10^6	$<10^2$	−
wt *Hin*dIII-E	2.4×10^6	$<10^2$	−
AR 5 BH 7500	4.5×10^6	2.6×10^4	+
AR 8 BH 7500	2.9×10^6	4.3×10^4	+
AR 9 BH 7500	4.8×10^6	7.5×10^4	+
AR 11 BH 7500	4.4×10^6	6.2×10^4	+
AR 12 BH 7500	5.1×10^6	4.9×10^4	+
AR 5 M 2700	3.2×10^6	1.9×10^4	+
AR 8 M 2700	3.3×10^6	3.3×10^4	+
AR 9 M 2700	4.7×10^6	4.1×10^4	+
AR 11 M 2700	3.4×10^6	7.0×10^4	+
AR 12 M 2700	2.6×10^6	1.8×10^4	+
AR 5 R 2900	5.5×10^6	2.4×10^4	+
AR 8 R 2900	2.1×10^6	2.7×10^4	+
AR 9 R 2900	5.8×10^6	7.9×10^4	+
AR 11 R 2900	3.7×10^6	4.0×10^4	+
AR 12 R 2900	3.6×10^6	2.8×10^4	+
AR 5 RHa 500	3.2×10^5	<5	−
AR 8 RHa 500	4.6×10^5	<5	−
AR 9 RHa 500	4.1×10^5	<5	−
AR 11 RHa 500	4.8×10^5	<5	−
AR 12 RHa 500	3.4×10^5	<5	−
AR 5 HaHi 500	3.7×10^5	9.1×10^2	+
AR 8 HaHi 500	2.3×10^5	2.9×10^2	+
AR 9 HaHi 500	3.5×10^5	3.5×10^2	+
AR 11 HaHi 500	2.5×10^5	1.7×10^2	+
AR 12 HaHi 500	3.0×10^5	5.3×10^2	+
AR 5 HiHa 500	4.5×10^5	<5	−
AR 8 HiHa 500	3.5×10^5	<5	−
AR 9 HiHa 500	4.4×10^5	<5	−
AR 11 HiHa 500	3.6×10^5	<5	−
AR 12 HiHa 500	2.5×10^5	<5	−
No DNA	2.8×10^5	<5	−
wt *Hin*dIII-E	3.2×10^5	<5	−

[a] DNA fragments are named according to the viral isolate from which they were cloned and their delimiting restriction sites. The location of the fragments within the *Hin*dIII E fragment is shown in Fig. 1. The first two lines represent negative controls for transfections involving the BH7500, M2700, and R2900 fragments. The last two lines represent negative controls for transfections involving the RHa500, HaHi500, and HiHa500 fragments. B, *Bam*HI; H, *Hin*dIII; M, *Mlu*I; R, *Eco*RI; Ha, *Hae*III; Hi, *Hin*cII.

[b] Infected cultures were transfected with the DNA fragments shown. Aphidicolin was added at 15 h p.i.; cells were harvested at 3 or 4 days p.i.

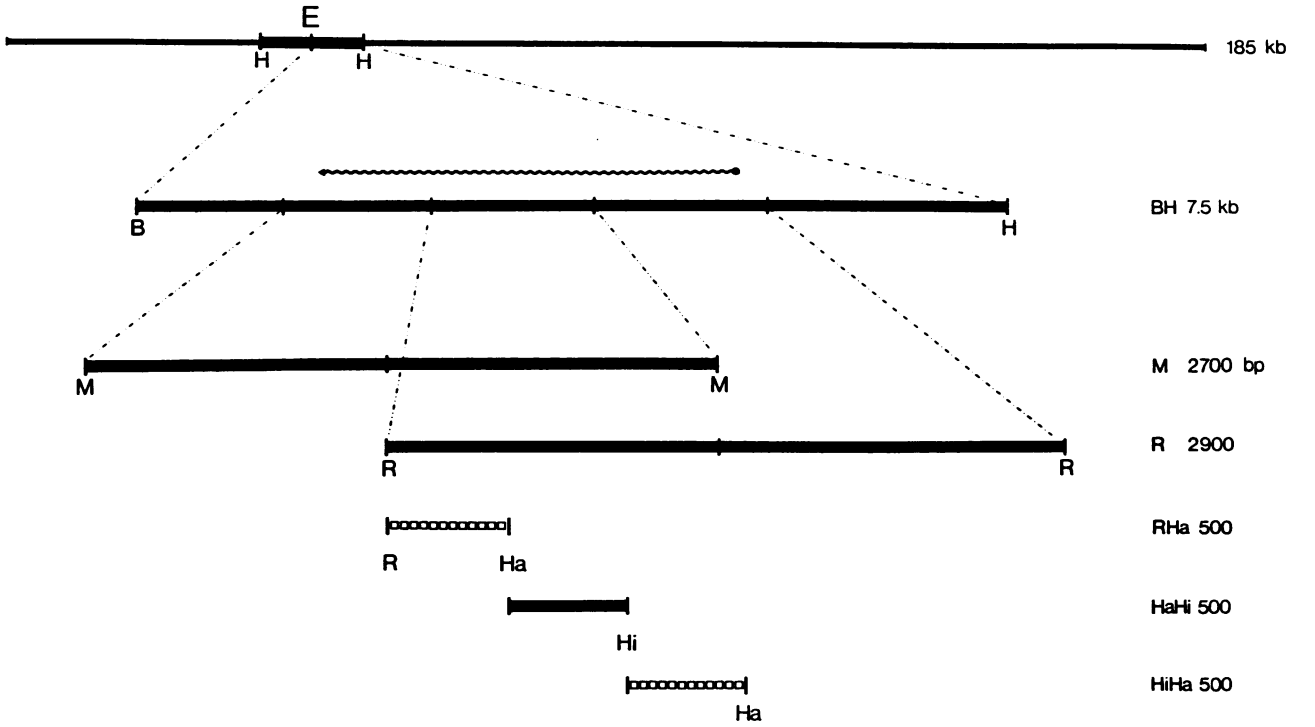

FIG. 2. Restriction fragments spanning the DNA polymerase gene used in marker rescue analysis. The complete vaccinia virus genome is indicated at the top, and the position of the mRNA for the DNA polymerase gene within the right-hand half of the genomic *Hin*dIII E fragment is indicated by the wavy line. Fragments derived from Aph^r isolates capable of conferring aphidicolin resistance are indicated by thick lines; fragments which could not confer resistance are indicated by open squares. DNA fragments are named by their delimiting restriction sites (from left to right with respect to the vaccinia genome) and by their sizes in base pairs. Restriction enzymes are abbreviated as follows: B, *Bam*HI; H, *Hin*dIII; Ha, *Hae*III; Hi, *Hin*cII; M, *Mlu*I; R, *Eco*RI.

ence of inhibitory concentrations of drug, and the frequency of large, resistant plaques was measured. Because these experiments were initiated before molecular analyses of the mutant lesions were complete, all five well-studied mutants were included in this study. These results are shown in Table 2. Comparison of the duplicate experiments indicates that the values obtained for each isolate were quite reproducible. The most striking result was the dramatic increase in mutant frequency apparent in the AR 9 stock. The other four stocks exhibited a range of mutation rates, with AR 8 most like wt, AR 11 and AR 12 showing similarly elevated frequencies of Rif^r, and AR 5 exhibiting increased frequencies of both Rif^r and IBT^r.

Observation of this range of phenotypes made us suspect that additional extra- or intragenic mutations might have been sustained in some or all of these individual isolates during the initial nitrosoguanidine treatment. To allow a rigorous examination of the impact of each Aph^r lesion on the virus, it seemed advantageous to isolate congenic viral strains which differed only in the polymerase lesion.

Isolation of congenic viral strains carrying the Aph^r lesions in an unmutagenized, wt background. Cultures were infected with wt virus and transfected with the minimal 500-bp *Hae*III-*Hin*cII fragment (Fig. 2 and Table 1) cloned from the genomic DNA of each Aph^r isolate. Plaque-purified Aph^r isolates were obtained from each infected-transfected culture. Further plaque purification and amplification were performed in the presence of drug for one plaque and in the absence of drug for a second to assess the stability of the Aph^r phenotype and to reveal any additional effects of the drug on the virus. For each genomic isolate and under each experimental condition, aphidicolin resistance was stable and comparable (data not shown). These stocks, designated ARP-5+, ARP-5, ARP-9+, etc. (aphidicolin-resistant polymerase), were used for all further studies.

Substitution of Val for Ala at residue 498 confers a mutator phenotype. The frequency of PAA^r, Rif^r, and IBT^r was measured for wt virus and for each congenic Aph^r strain. To control for variations in mutation rate which might accompany infections performed at different multiplicities, a wt stock prepared in parallel with the congenic Aph^r stocks was used. The results are shown in Table 3. It is clear that ARP-9 (Ala-to-Val mutation) stocks again showed a significantly increased frequency of PAA^r, Rif^r, and IBT^r mutants; the increase for the last two drugs, which do not interact with the DNA polymerase, is higher than that seen for PAA. The rate of mutation appeared somewhat higher in the stock amplified in the presence of drug, especially regarding the generation of PAA^r mutants. The other Aph^r strains (Ala-to-Thr mutations) appeared to have mutation rates indistinguishable from those of the wt virus. The constant values obtained with the last four congenic strains, shown subsequently by sequence analysis to contain identical Aph^r lesions, strengthened our confidence in the significance of the elevated mutation rate observed in the ARP-9 stocks. In addition, comparison of this constancy with the variability observed during analysis of the original AR 5, 8, 11, and 12 strains (Table 2) provided further support for the conclusion that these mutants, although bearing identical Aph^r lesions, were independent isolates.

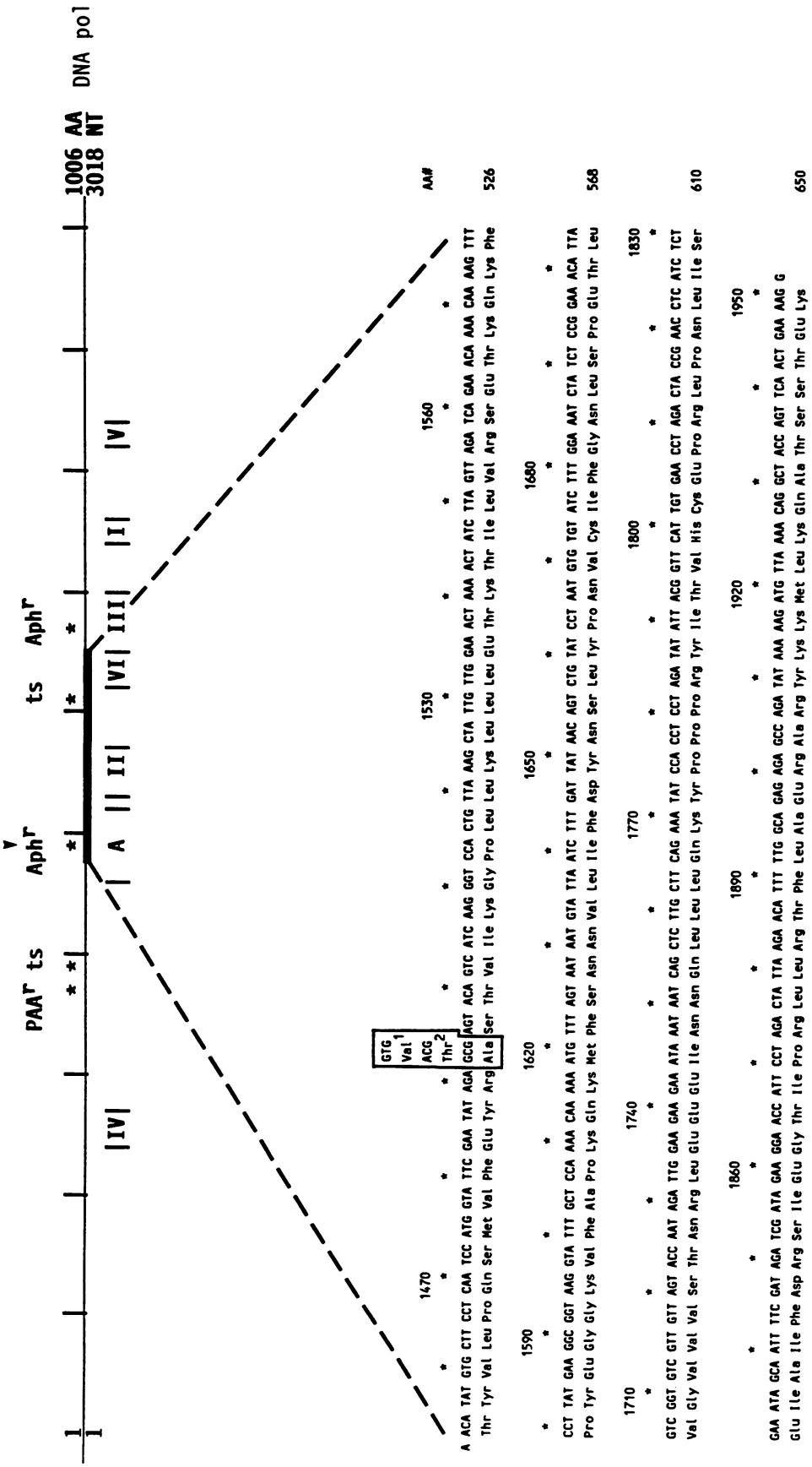

FIG. 3. Localization of nucleotide changes responsible for aphidicolin resistance. The vaccinia virus DNA polymerase-coding region is diagrammed at the top. Roman numerals and the letter A beneath the line indicate the relative locations of regions conserved between the vaccinia virus enzyme and other DNA polymerases (15, 43, 44). Above the line are the relative positions of known point mutations (asterisks) and their associated phenotypes (Aph^r [10; this manuscript], PAA^r [12, 38, 39], and temperature sensitivity [ts] [38, 39]). The location of the mutations described in this report is indicated by an arrowhead. The thick line indicates the 500-bp *Hae*III-*Hin*cII fragment which conferred Aph^r by marker rescue (Fig. 2 and Table 1). The sequence of this fragment was determined for each mutant as described in Materials and Methods and is shown at the bottom. The single-nucleotide changes observed in the mutant alleles are indicated above the wt sequence and are highlighted by a box. 1, Mutation conferring Aph^r in AR 9; 2, mutation conferring Aph^r in AR 5, 8, 11, and 12.

TABLE 2. Comparison of spontaneous mutation frequencies in aphidicolin-resistant and wt viral stocks[a]

Virus	Mutation frequency								
	PAA[r][b]			Rif[r]			IBT[r]		
	Assay 1	Assay 2	Relative to wt[c]	Assay 1	Assay 2	Relative to wt	Assay 1	Assay 2	Relative to wt
WT	2.4	2.8	1.0	3.2	3.0	1.0	17.0	19.0	1.0
AR 5	11.0	12.0	4.6	87.0	49.0	21.9	510.0	670.0	32.8
AR 8	1.6	3.5	1.0	8.6	6.4	2.4	62.0	86.0	4.1
AR 9	52.0	49.0	19.6	600.0	360.0	154.8	19,000.0	11,000.0	833.3
AR 11	5.9	6.9	2.5	52.0	32.0	13.6	78.0	75.0	4.3
AR 12	4.8	5.8	2.0	72.0	96.0	27.1	60.0	95.0	4.3

[a] Each stock was titrated in the absence of drug and in the presence of PAA at 200 μg/ml, rifampin at 150 μg/ml, or IBT at 60 μM. Two independent assays were performed.
[b] Per 10^5 PFU.
[c] The average of the two experiments was compared with the average for wt.

Comparison of DNA synthesis and viral yield following infection with wt, ARP-9, and ARP-12. For subsequent phenotypic studies, comparisons were made between the plaque-purified wt stock, ARP-9, and one representative of the Ala-Thr class, ARP-12. To quantitate the degree to which each lesion conferred aphidicolin resistance, viral yield at 24 h p.i. was determined following infection at an MOI of 1 in the absence or presence of 85 μM aphidicolin. As shown in Fig. 4A, wt yield in the presence of drug was only 0.01% of that obtained in untreated cultures. In infections performed with either ARP-9 or ARP-12, aphidicolin exerted only a minimal inhibition, reducing viral yields to 25% of those obtained in untreated cultures. Thus, the degree of biological resistance conferred by each mutation was comparable and impressive. A more biochemical measure of the resistance of the DNA polymerase itself to aphidicolin inhibition was obtained by quantitating viral DNA synthesis following infections with the same viral stocks at an MOI of 5. As shown in Fig. 4B, the profile of accumulation of viral DNA sequences in cultures infected with wt, ARP-9, or ARP-12 in the absence of drug was indistinguishable. Thus, the amino acid substitutions at residue 498 had no discernible impact on the rate or duration of DNA synthesis per se. In the presence of drug, wt virus demonstrated a virtual absence of DNA synthesis, as expected. In contrast, ARP-9- and ARP-12-infected cultures synthesized significant amounts of viral DNA, albeit at a somewhat slower rate. The levels of viral DNA synthesized were 30 to 50% of those attained in the absence of drug, a result comparable to that observed for viral yield.

TABLE 3. Comparison of spontaneous mutation frequencies in congenic aphidicolin-resistant and wt viral stocks

Virus[a]	Mutation frequency relative to that of wt[b]		
	PAA[r]	Rif[r]	IBT[r]
wt	1	1	1
Ala-Thr			
ARP-5+	1.5	1.3	2.3
ARP-5	0.5	0.4	0.9
ARP-8+	1.9	1.8	3.4
ARP-8	0.5	0.4	1.1
ARP-11+	1.4	0.8	2.6
ARP-11	0.7	0.7	1.1
ARP-12+	0.6	1.0	1.8
ARP-12	0.3	1.8	0.9
Ala-Val			
ARP-9+	15.8	25.7	44.4
ARP-9	6.0	21.7	18.5

[a] The preparation and nomenclature of the wt and congenic aphidicolin-resistant strains are described in Materials and Methods.
[b] To determine the frequency of drug resistance, each stock was titrated in the absence of drug and in the presence of PAA (200 μg/ml), rifampin (150 μg/ml), or IBT (60 μM).

ARP-9 and ARP-12 show different degrees of hypersensitivity to PAA, araC, and araA. Pharmacological and genetic analyses of HSV DNA polymerase have revealed that resistance to inhibitors presumed to interact with catalytic residues is frequently associated with cross-resistance or hypersensitivity to other such inhibitors (2, 5–7, 11, 19–21, 25, 26, 31, 41). Therefore, the sensitivities of ARP-9 and ARP-12 to PAA, araC, and araA were investigated. PAA is thought to interfere with the exchange of PP_i and deoxynucleoside triphosphates within the nucleotide-binding domain of the polymerase; araC and araA are nucleoside analogs of cytosine and adenosine, respectively. Initially, plaque assays of wt, ARP-9, and ARP-12 stocks were performed in parallel in the presence of increasing concentrations of each drug. Comparison of plaque sizes in the presence of various concentrations of PAA or araA revealed that ARP-9 was significantly hypersensitive to these drugs and ARP-12 was mildly hypersensitive (data not shown). Both mutants showed a moderate hypersensitivity to araC. These phenotypes were further evaluated by measuring the viral yield obtained at 24 h p.i. with wt, ARP-9, or ARP-12 at an MOI of 1 in the presence of several concentrations of PAA, araC, or araA. These results are presented in Fig. 5.

The extreme hypersensitivity of ARP-9 to PAA is evident; at a drug concentration of 50 μg/ml, ARP-9 yield was reduced to 0.02% of the control value, whereas wt yields were diminished only to 30%. At 100 μg of PAA per ml, ARP-12 yield was reduced to 0.4% of the control value, whereas wt was 10-fold less sensitive and 4% of control levels of virus were harvested. ARP-9 and ARP-12 showed similar hypersensitivities to araC; in the presence of various concentrations of drug, inhibition of the mutant stocks was 10- to 25-fold more severe than that observed for wt stocks. (Comparison of these two assays of drug sensitivity revealed that macroscopic plaques were no longer apparent when viral yield dropped below 2% of control levels.) In the case of araA, ARP-9 demonstrated a 400-fold hypersensitivity to 2 μM drug, with a reduction in yield to 0.04% of the control value, whereas the wt yield was reduced only to 16%. In the

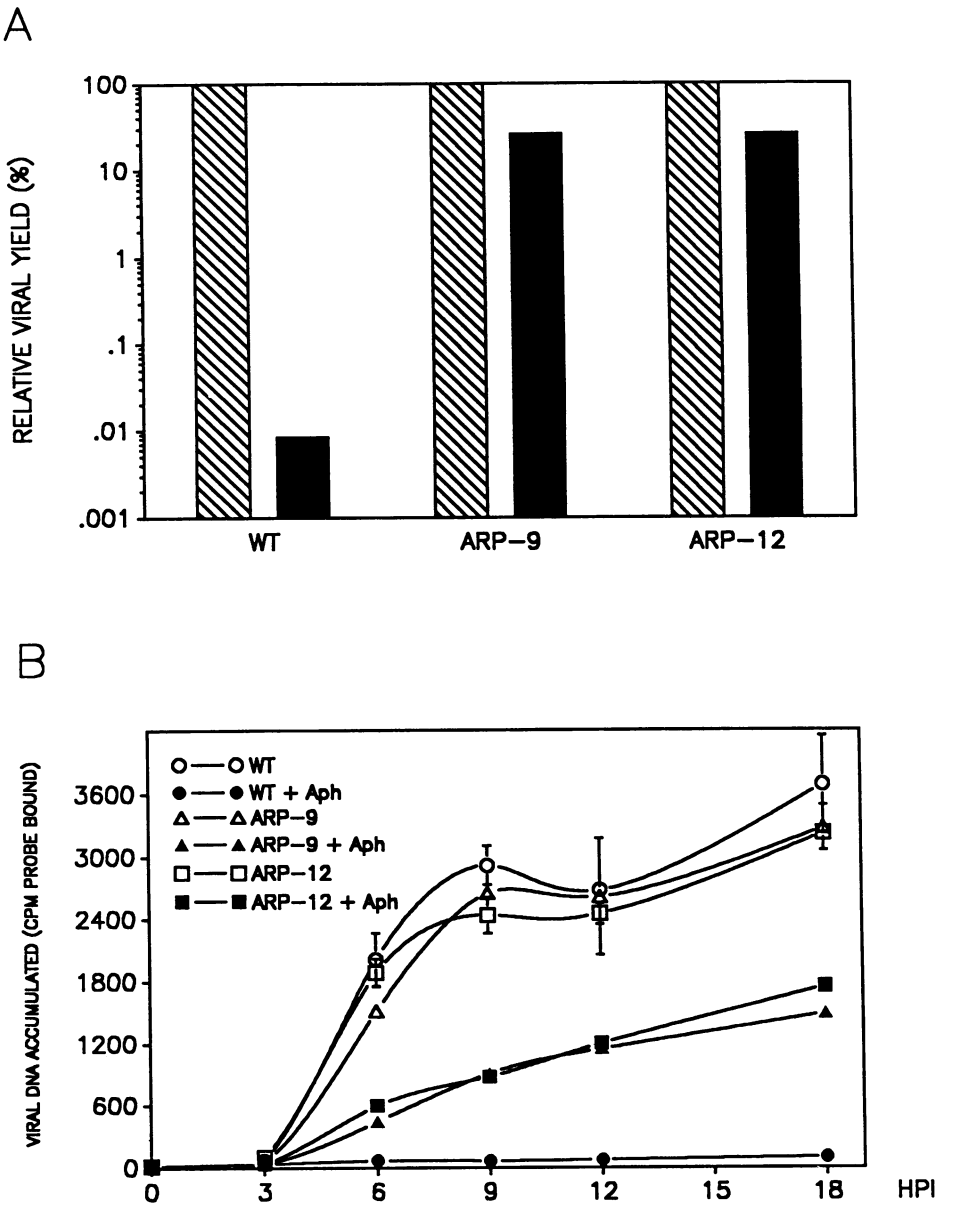

FIG. 4. (A) Effect of aphidicolin on 24-h viral yield from cultures infected with wt or congenic Aph^r viruses. BSC40 cells were infected at an MOI of 1 and fed with medium containing no drug (▨) or 85 μM aphidicolin (■). Cells were harvested at 24 h p.i. as described in Materials and Methods, and viral yields were determined by plaque assay in the absence of drug. The yield obtained in the presence of drug is expressed as a percentage of that obtained in the absence of drug. (B) DNA synthesis following infection with wt or congenic Aph^r viruses. BSC40 cells were infected at an MOI of 5 and fed with medium containing 85 μM aphidicolin or no drug. Whole-cell lysates were prepared at various times p.i., bound to filters, and probed with radiolabeled vaccinia virus DNA. The radioactivity bound to each sample was quantitated and plotted graphically; the abscissa represents hours p.i. (HPI), and the ordinate represents counts per minute of probe bound to each sample.

presence of 4 μM araA, ARP-12 was 25-fold more sensitive than was wt, showing a reduction in yield to 0.03% of control values in comparison with the observed recovery of 0.67% of control levels of wt virus.

DISCUSSION

In this report, we describe the isolation of Aph^r isolates of vaccinia virus. The lesions in the mutants were mapped by identifying the minimal fragment of each cloned polymerase allele capable of conferring drug resistance on wt virus by homologous recombination; ultimately, the nucleotide sequence of a relevant 500-bp fragment was obtained for each isolate. (In retrospect, the lesion in each isolate was only 41 or 42 nucleotides from the terminus of the 500-bp fragment, illustrating that, at a maximum, the vaccinia virus enzymatic machinery requires only this short flanking homology to direct accurate and efficient recombination.) Although the virus inoculum used to generate the Aph^r isolates was predicted to contain more than 100 independent mutants,

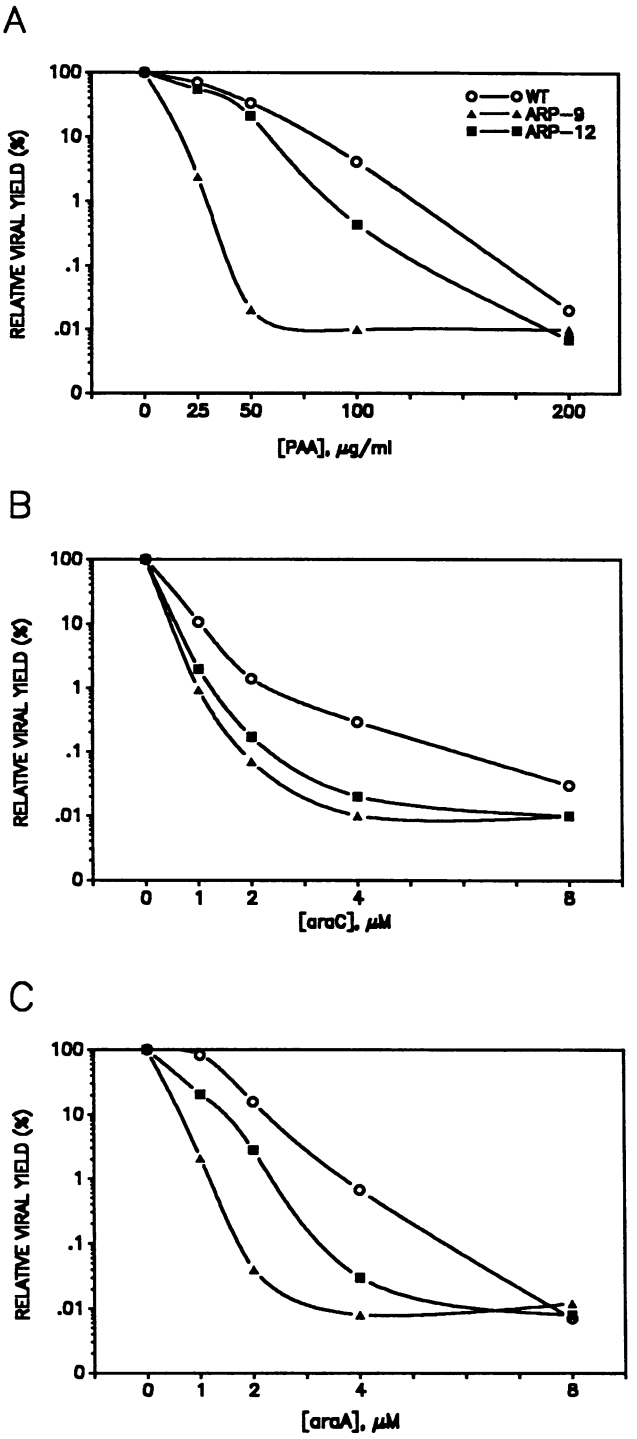

FIG. 5. Hypersensitivity of congenic Aph^r viruses to PAA, araC, and araA. BSC40 cells were infected with the indicated virus at an MOI of 1 and fed with media containing various concentrations of PAA (A), araC (B), or araA (C). Cells were harvested at 24 h p.i. as described in Materials and Methods, and viral yields were determined by plaque assay in the absence of drugs. The yield obtained in the presence of drug is expressed as a percentage of that obtained in the absence of drug.

sequential passaging of this inoculum in the presence of drug would have selected for those Aph^r viruses exhibiting rapid replication and maximal drug resistance. However, phenotypic variations among the plaque-purified Aph^r stocks suggests that several independent isolates were recovered. Nevertheless, 9 of 10 stocks carried the same nucleotide substitution within the DNA polymerase gene, and the 10th stock contained a distinct substitution in the same codon (amino acid 498). This finding suggests that this residue is intimately involved in the binding of aphidicolin, and presumably dCTP, to the DNA polymerase and, moreover, that production of aphidicolin resistance by transitional mutations is most likely to involve changes at this codon. It is worth noting that the two substitutions obtained, replacement of an Ala residue by Thr or Val, are the only possible outcomes of transitional mutations in this codon. The only other Aph^r isolate of vaccinia virus (9, 10) maps elsewhere in the polymerase gene (codon 670) and was created by a transversional mutation. Transitional mutations at this distal residue either are silent or lead to the substitution of a Pro for a Leu residue, a severe change which might be incompatible with enzymatic activity and virus viability. The observation that lesions at such distant codons can generate aphidicolin resistance supports the presumption that tertiary interactions between distal portions of the polypeptide are involved in generating the nucleotide-binding site.

That the amino acid substitutions are not deleterious to DNA replication or virus production per se was evidenced by the fact that, in the absence of aphidicolin, wt and congenic Aph^r stocks induced indistinguishable profiles of DNA synthesis (Fig. 4B) and produced equivalent yields of infectious progeny during a 24-h, low-multiplicity infection (data not shown). Similarly, the Aph^r phenotype was stable and persisted in viral stocks passaged in the absence of selective pressure. The two amino acid substitutions seemed equally effective in establishing viral resistance to aphidicolin. Both the rate of DNA synthesis and the final levels of sequences accumulated were moderately and equivalently diminished (to approximately 30 to 50% of control levels) when cultures were infected with the mutants in the presence of 85 μM aphidicolin, and viral titers were 25% of those obtained in the absence of drug. In contrast, this level of drug abrogated any detectable DNA synthesis in cultures infected with wt virus, and only 0.01% of control levels of virus were released from these cultures.

The residue affected in the mutants does not lie within any of the most highly conserved regions of the polymerase sequence. It does, however, fall within the A region that is common to poxvirus and herpesvirus polymerases and that flanks residues thought to play a role in nucleotide binding (15). That the mutations which affect aphidicolin resistance alter the nucleotide-binding domain is supported by the observation that both classes of mutants recovered are hypersensitive to the nucleotide analogs araC and araA. Genetic selection for resistance to aphidicolin, which acts as a competitive inhibitor with respect to dCTP, might be expected to yield mutations which alter the conformation of the nucleotide-binding domain as it is poised to bind a dCTP residue. In addition to reducing aphidicolin binding, these changes could differentially affect the K_m of the enzyme for dCTP and araCTP, leading to hypersensitivity to the nucleotide analog. It is not immediately apparent why the conformational changes conferring aphidicolin resistance should affect, or more specifically reduce, the ability of the DNA polymerase to distinguish between correct and incorrect sugar moieties within nucleotide substrates. However, al-

tered sensitivity to nucleotide analogs has been a frequent observation in studies of other Aph^r polymerases (20, 29, 31). Since the Ala-Thr and Ala-Val mutations in codon 498 of the vaccinia virus DNA polymerase confer equivalent resistance to aphidicolin, it is not unreasonable that they confer equivalent hypersensitivity to araC.

Both Aph^r alleles are also hypersensitive to araA, suggesting that binding of both purines and pyrimidines is altered by the substitution at residue 498. Hypersensitivity to PAA, a pyrophosphate analog, was also observed. PAA is thought to inhibit DNA polymerases by acting uncompetitively with respect to DNA and noncompetitively with respect to nucleotides. However, studies with HSV DNA polymerase in vitro have demonstrated that inhibition by nucleotide analogs, aphidicolin, and phosphonoformic acid (a congener of PAA) is mutually exclusive, suggesting that the binding sites for these inhibitors do overlap (14). The altered sensitivity to PAA seen in our mutants is consistent with this conclusion. Indeed, an inverse relationship between Aph^r and PAA^r (or PFA^r) has been observed repeatedly in analyses of other DNA polymerases (2, 6, 19–21, 29, 31, 41). It is worth noting that the previously mapped PAA^r mutation in vaccinia virus maps to codon 372 (12, 38, 39), suggesting again that distal residues are likely to contribute to the tertiary structure of the enzyme's active site. Relative to the Ala-Thr mutation, the Ala-Val substitution confers significantly greater hypersensitivity to araA and PAA. This observation suggests that the conformation of the nucleotide-binding domain as it is poised to bind dATP, as well as the phosphate-binding portion of the domain, are more distorted by the incorporation of a Val in position 498.

Interestingly, it is also the Val substitution which confers a mutator phenotype. Congenic viral strains bearing this single amino acid alteration exhibited 20- to 40-fold increases in spontaneous mutation frequency as measured by quantitations of Rif^r and IBT^r viruses within similarly prepared wt and Aph^r stocks. The frequency of PAA^r viruses was elevated 6- to 16-fold. (This more modest increase may reflect the observed hypersensitivity of the Aph^r stocks to PAA. In essence, the 200 μg of PAA per ml used to quantitate resistance exerts a threefold-greater inhibitory effect on the Val-containing mutant than on wt virus. Even if the concentration of PAA was lowered to adjust for the hypersensitivity of the stock, it might be inherently more difficult to generate PAA^r within the Aph^r background; it must be remembered that these studies are performed in vivo and that alterations in the DNA polymerase must not have adverse affects on polymerase activity or virus viability. Previous investigators have experienced difficulty in isolating PAA^r derivatives of an Aph^r HSV-2 strain that demonstrated collateral hypersensitivity to PAA. Those PAA^r variants eventually isolated had lost their Aph^r and gained a variety of altered resistances to nucleotide analogs [31].) That the appearance of drug-resistant viral progeny reflects bona fide mutational events rather than amplification of genes encoding the targets of the specific drugs is supported by our inability to detect any variation in the molarity or mobility of restricted genomic DNA fragments isolated from the mutant stocks (data not shown).

There is ample precedent for an association of altered polymerase fidelity with altered polymerase sensitivity to drugs that interact with the nucleotide-binding domain. Mutator and antimutator isolates of HSV-1, HSV-2, and CHO cells have been associated with structural changes in the DNA polymerase nucleotide-binding domain (17, 20, 28, 31, 32, 41). Mutations which decrease the K_m for a given nucleotide can increase the mutation rate, whereas increases in K_m can decrease the mutation rate. Presumably, a decreased K_m for nucleotide substrates would allow even the incorrect nucleotide to bind well enough to be incorporated into the growing chain, whereas increases in K_m would select for stringent nucleotide selection. A 10-fold decrease in the K_m for dCTP was observed in an Aph^r DNA polymerase α isolated from Aph^r CHO cells; these cells displayed a 6-fold elevation in their spontaneous-mutation rate (28). An antimutator DNA polymerase studied in HSV-1 caused an approximately 50-fold decrease in the spontaneous mutation rate and a 2-fold increase in K_m for dATP, dCTP, and TTP (17). The araC hypersensitivity observed in mutants bearing either of our Aph^r alleles suggests that these mutations may differentially alter the K_m of the enzyme for dCTP and araCTP. The mutator phenotype conferred by the Val substitution suggests that this alteration may decrease the K_m for the other three deoxynucleoside triphosphates far more significantly than does the Thr substitution. This possibility is consistent with the differential hypersensitivity to araA observed in the two mutants.

Biochemical confirmation of these predictions awaits the overexpression and purification of the mutant DNA polymerase molecules. The studies described in this report shed new light on the pharmacological properties of the vaccinia virus DNA polymerase and suggest that the vaccinia virus enzyme may be a good model system for probing cross-resistance and mutually exclusive drug resistance. In addition, these genetic findings provide clues as to the residues of the enzyme involved in forming the nucleotide-binding domain and offer an intriguing system for probing the basis of polymerase fidelity.

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